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Submitted as a Technical Note

Characterisation and cross-species utility of 20 microsatellite markers for population and forensic applications in the endangered Carnaby's Black-cockatoo, *Calyptorhynchus latirostris*

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Abstract

We characterise 20 microsatellite loci identified from the endangered Carnaby's Black-cockatoo (*Calyptorhynchus latirostris*). The primers were tested across 40 individuals from the southwest of Western Australia and displayed between 4 and 11 alleles per locus with expected heterozygosities ranging from 53 to 87% and exclusion probabilities of ≥0.999. These loci will be useful in population genetic studies to facilitate conservation management decisions in addition to wildlife enforcement applications for the endangered Carnaby's Black-cockatoo. We also tested the markers in 12 high profile and smuggled species from five genera, *Cacatua*, *Callocephalon*, *Calyptorhynchus*, *Nymphicus* and *Probosciger*. These species detected between two to 19 alleles per locus with 50 to 100% amplification success.

Keywords: Cacatuidae, Carnaby's Black-cockatoo, microsatellites, population genetics, wildlife forensics

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The endangered Carnaby's Black-cockatoo (*Calyptorhynchus latirostris*), also known as the Short-billed Black-cockatoo is one of 19 bird species endemic to the southwest corner of Western Australia, an internationally recognised biodiversity hotspot (Myers et al. 2000). The species has suffered a 50% reduction in its range and dramatic declines in population size since the 1960's. This iconic species is threatened by a combination of illegal poaching and shooting, habitat loss, competition for nest hollows and food resources (Garnett and Crowley 2000; Saunders and Ingram 1995). To increase our knowledge and facilitate more informed decision-making by wildlife managers and enforcement officers, this study was undertaken and describes the variability of 20 highly polymorphic microsatellite loci for *C. latirostris* and cross-species utility for a large number of other cockatoo species of conservation concern.

A microsatellite library was developed from one deceased individual *C. latirostris* collected by the Department of Environment and Conservation of Western Australia (Western Australian Museum, WAM 37011). We extracted total genomic DNA from liver tissue by standard desalting: tissue digestion in cell lysis buffer (0.1 M Tris-HCl pH 8.0, 0.01 M NaCl, 0.1 M EDTA, 0.5% SDS) for nucleated blood (Longmire et al. 1997) with proteinase K (Invirtogen), 4M ammonium acetate precipitation, and a final precipitation using ethanol prior to resuspending the DNA in TE buffer (10 mM Tris, 0.1 mM EDTA, pH8). Sixty-seven clones from four genomic libraries (enriched for

repetitive regions CA, ATG, CATC and TAGA; GenBank accession numbers GQ358643 – GQ358709) were created by Genetic Identification Services (http://www.genetic-id-services.com/index.htm). We used Primer Express 2.0 (Applied Biosystems) to design primers, of which the forward primer was labelled with an M13-tag at its 5'-end as a cost effective approach to screening large numbers of primer sets and described by Schuelke (2000). DNA fragments were separated on an Applied Biosystems 3730 DNA Analyser. Size was determined by co-running a size standard (Genescan™-500 LIZ™; Applied Biosystems, Melbourne) and fragments were scored manually with the aid of GeneMarker™ Software (Soft Genetics). All monomorphic loci were discarded and only the M13-tag primers for polymorphic loci were then exchanged for fluorescently labelled tags (Applied Biosystems).

Each singleplex (25 µL) PCR contained approximately 50 ng DNA, 1 X PCR buffer, 2.5 mM $MgCl_2$, 400 µM each dNTP, 0.1 mg BSA, 0.16 µM of each primer, and 2.5 units of *Taq* polymerase (Fisher Biotec). Parameters for thermal cycling were as follows: 95 °C for 3 min followed by 40 cycles at 95 °C for 30 s, 60 °C for 45 s and 72 °C for 45 s, followed by 72 °C for 10 min. Genotypic data were initially manipulated using Microsoft Excel, were checked for errors and input files were created for other programs. Descriptive statistics (number of alleles, observed and expected heterozygosities), deviations from Hardy-Weinberg equilibrium (HWE) and total exclusionary powers (*PE1, PE2;* Jamieson and Taylor 1997) were generated using GenAlEx 6.2 (Peakall and Smouse 2006). Linkage disequilibrium was assessed using GENEPOP (Raymond and Rousset 1995) and the frequency of null alleles (p_{null}) was estimated within the program ML-Relate (Kalinowski and Taper 2006, Kalinowski et al. 2006). Loci were assigned to a chromosome location in the zebra finch (*Taeniopygia guttata*) genome (compiled by the Washington University School of Medicine Genome Sequencing Centre) based on sequence homology using the program BLASTN 2.2.21 (Altschul et al. 1997; http://blast.ncbi.nlm.nih.gov/Blast.cgi) which uses a

heuristic algorithm able to detect relationships among sequences that share only isolated regions of similarity.

A total of 20 microsatellite loci were optimised (Table 1) and showed between 4 and 11 (mean=6.650 ± 0.443) alleles per locus, with observed and expected heterozygosities ranging from 0.400 - 0.825 (mean=0.635 ± 0.032) and 0.535 - 0.865 (mean=0.699 ± 0.021), respectively. Eighteen of 20 loci were assigned to a chromosome location (Table 1) and locus p*Cl*B11 was found to be Z-linked therefore only males were described, as females were homozygous. Two loci p*Cl*D108 and p*Cl*D118 did not conform to Hardy-Weinberg equilibrium and were found to have a frequency of null alleles (p_{null}) greater than >0.20, although there may be other reasons for observing a heterozygote deficiency at these loci such as inbreeding or Wahlund effect (Dakin and Avise 2004). Linkage disequilibrium was detected with loci p*Cl*D8 and p*Cl*D119, which were subsequently removed from further analysis, and were not assessed for cross-species utility. Total exclusionary powers ≥0.999 was reached at nine locus combination, irrespective of when only one parent or both parents were known (Jamieson and Taylor 1997). These markers demonstrate their utility for population genetic studies, making them suitable for detecting illegal trade (Manel et al. 2002) and providing extremely useful genetic resolution to contribute to the overall conservation, management and protection of an internationally recognised, endangered and iconic cockatoo species.

A total of 180 individuals from five genera were assessed for cross-species utility and presented in Table 2. The number of alleles per locus ranged from two to 19 for the *Calyptorhynchus* genus, two to 11 alleles per locus for *Callocephalon* and *Cacatua*, two to nine alleles for *Nymphicus* and two to four alleles were identified for *Probosciger* (Table 2). The amplification success rate for polymorphic loci identified in related cockatoo species ranged from 50 to 100 per cent (Table 2),

therefore these microsatellite markers may facilitate future conservation genetic studies while reducing the associated time and costs of development.

Acknowledgements

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Table 1 Description of 20 variable microsatellite loci isolated from the Carnaby's Black-cockatoo (*Calyptorhynchus latirostris*)

Locus	Repeat motif	Chromosome and location (bp)	Primer sequence 5′-3′ (including the fluorescent label)	*N*	N_A	Size range (bp)	H_O	H_E
p*Cl*A119	$(GT)_{21}$	Tgu7 (8743875)	F:[FAM]TGACACTTTCCTGTGGCTGC R: ATTACTTTGTTATTTCCACTGCTTGC	39	11	89 – 117	0.538	0.554
p*Cl*A125	$(CA)_{20}$	NSH	F:[VIC]GCTTAGCGAACATTAAATCTGCAC R: TCAGGTTTCCTGAAGAGAAACCAG	40	11	115 – 139	0.775	0.865
p*Cl*A139	$(CA)_{18}$	Tgu6 (8681202)	F: GTTGCTAAGATTGGATAACACCAGATT R:[FAM]ATAAGTTGCAGTTTGTACGCGC	40	8	167 – 185	0.700	0.724
p*Cl*A105	$(CA)_{15}$	Tgu14 (1626425)	F:[PET]TCCCATCCACCCCATGC R: TCATGTGTTCTTGCCCAGTTTG	40	8	107 – 123	0.725	0.745
p*Cl*A9	$(GT)_{15}$	Tgu7 (1105022)	F:[NED]GCTGCAGAACATGGTCACATTC R: ACTCTGATAACTCAACATTGCCCA	40	7	74 – 92	0.650	0.580
p*Cl*A118	$(GT)_{15}$	Tgu1 (9293668)	F:[VIC]GAGTTAAAAAAGACCCAAACCCAA R: TGTATTGTGTTAAGATGATGCATGACA	40	5	119 – 127	0.750	0.722
p*Cl*A128	$(AC)_{13}$	Tgu1 (9454118)	F:[FAM]TGGAATATACATAAAAATTTGTTACAGGG R: TTGTTTGCTTTTATAACAGAGAGAGAATG	19	4	104 – 110	0.632	0.719
p*Cl*A138	$(GT)_{12}$	Tgu3 (17204322)	F:[FAM]ATGAGACCAAAGGTCTTGGGTG R: TGCAATCAGATGCTGGAATCA	40	4	174 – 188	0.650	0.568
p*Cl*B11	$(ATG)_{16}$	TguZ (7531463)	F:[FAM]CTGCTTTTGTTGCCTCTAAC R: ATGCCCTATGATGAGATGC	20 (Males)	7	199 – 230	0.850	0.749
p*Cl*D108‡	$(TAGA)_{15}$	Tgu11 (1883671)	F:[FAM]ATCCTGCTGTGTTTAGAAAATACTTTGTAAT R: TCAGATATTTACATAACAGCCAGACTTG	39	7	214 – 242	0.385	0.819
p*Cl*D122	$(TAGA)_{14}$	Tgu3 (1470384)	F:[NED]AAAGTCACTTGGACTGAGGAGCTC R: CATTTCTGAATAGTTTTAGTAGCTCTTCCTG	40	8	114 – 142	0.800	0.748

p*Cl*D118‡	$(TAGA)_{13}$	NSH	F:^{VIC}CTATTGACTAGGAGATCTACAAGCCGT	38	7	187 – 211	0.421	0.792
			R: TGCACCCCTCTTCTGCATC					
p*Cl*D7	$(TAGA)_{12}$	Tgu2	F:^{PET}AGATCTCATTACTGCTTTGCATGATAG	40	7	88 – 120	0.750	0.759
		(27449558)	R: TTGCAACTGCTTCTGAAACTGC					
p*Cl*D105	$(ATAG)_{10}$	Tgu5	F:^{FAM}TCCCCACAACCACAGGACA	39	6	83 – 115	0.744	0.772
		(22806466)	R: CATGAAACATCCAAGAGCAATACAA					
p*Cl*D109	$(TCTA)_{10}$	Tgu10	F:^{VIC}TGAGAGCATAAATCAAGAAGGCAT	40	6	168 – 188	0.775	0.675
		(5810806)	R: AGCAGAGCTCCTGCATATTGG					
p*Cl*D114	$(CTAT)_{9}$	Tgu3	F: GTCTTGTATTAAATCACTGCAGAGAACTGT	40	5	120 – 136	0.550	0.570
		(4409470)	R:^{FAM}ATGATACACTGGGAGCTGGGAG					
p*Cl*D112	$(ATCT)_{8}$	Tgu20	F:^{NED}GCAGATGCAAATGTTACAGCAAC	40	5	161 – 179	0.600	0.683
		(1346575)	R: AACTAAATGGATATATTAAATGGAGAAATAGATG					
p*Cl*3*	$(AC)_{11}$	Tgu3	F:^{NED}TGCAAAATAAACTTGTCCCAGATG	40	6	118 – 134	0.825	0.714
		(5594880)	R: GAGACGTGAGAAATGGGTTCTGT					
p*Cl*5*	$(CA)_{13}$	Tgu17	F:^{NED}GTGCAGGGACCACTTGTATG	40	5	164 – 190	0.400	0.535
		(2552480)	R: CACATGGAGCATGAGCCC					
p*Cl*8*	$(GGAT)_{13}$	Tgu18	F:^{PET}GTCCTCCGAATAACCTGTGCA	38	5	128 – 144	0.605	0.660
		(2246152)	R: TGCTAAAAAGGTGGTAACACAGTGTC					

For each locus (GenBank accession numbers: GQ358643 - 661), we list the core repeat motif (from the original clone), loci that could be assigned a chromosome location in the zebra finch genome, locus p*Cl*B11 is Z-linked and only males described , no strong hits (NSH) for loci p*Cl*A125 and p*Cl*D118, primer pair (including the labeled fluorophore), number of individuals genotypes (N), number of alleles at each locus (N_A), size range (bp), observed (H_O) and expected (H_E) heterozygosity, ‡loci showed deviation from Hardy-Weinberg equilibrium, *primers (GenBank accession numbers AY775326 – 29) redesigned for this study McInnes et al. 2005.

table2
Click here to download table: Table2.doc

Table 2 Cross-species amplification of microsatellite loci developed for the Carnaby's Black-cockatoo (*Calyptorhynchus latirostris*)

Locus	Baudin's Black-cockatoo (*Calyptorhynchus baudinii*) (n = 24)	Yellow-tailed Black-cockatoo (*Calyptorhynchus funereus*) (n = 17)	Red-tailed Black-cockatoo (*Calyptorhynchus banksii*) (n = 45)	Glossy Black-cockatoo (*Calyptorhynchus lathami*) (n = 13)	Gang-gang cockatoo (*Callocephalon fimbriatum*) (n = 14)	Palm cockatoo (*Probosciger aterrimus*) (n = 3)	Sulphur-crested cockatoo (*Cacatua. galerita*) (n = 14)	Little corella (*Cacatua sanguinea*) (n = 10)	Western Long-billed corella (*Cacatua pastinator*) (n = 10)	Major Mitchell's cockatoo (*Cacatua leadbeateri*) (n = 12)	Galah (*Cacatua roseicapilla*) (n = 6)	Cockatiel (*Nymphicus hollandicus*) (n = 12)
p*Cl*A119	7 (95-117)	7 (93-117)	10 (89-115)	1 (105)	1 (99)	1 (85)	3 (89 – 93)	5 (87 – 109)	4 (87 – 109)	2 (89 – 91)	1 (85)	2 (105 – 115)
p*Cl*A125	7 (115-133)	10 (111-137)	8 (111-139)	3 (117-121)	3 (119-135)	1 (107)	3 (105 – 113)	8 (107 – 125)	7 (111 – 125)	2 (109 – 113)	4 (111 – 119)	1 (107)
p*Cl*A139	4 (169-181)	10 (161-191)	7 (169-185)	1 (173)	1 (150)	2 (169-171)	1 (150)	-	1 (150)	-	1 (150)	3 (161 – 165)
p*Cl*A105	5 (109-121)	9 (113-139)	9 (107-123)	6 (99-143)	10 (109-139)	4 (99-115)	7 (99 – 121)	6 (109 – 129)	5 (105 – 123)	1 (103)	7 (105 – 121)	3 (98 – 102)
p*Cl*A9	5 (76-84)	8 (76-96)	-	2 (66-82)	-	-	-	-	-	2 (58 – 64)	2 (64 – 66)	1 (62)
p*Cl*A118	5 (117-125)	6 (109-123)	5 (107-127)	3 (107-119)	1 (97)	2 (111-113)	2 (81 – 91)	4 (79 – 99)	4 (79 – 99)	2 (93 – 99)	1 (95)	4 (99 – 107)
p*Cl*A128	4 (100-108)	4 (104-110)	6 (92-108)	1 (107)	4 (107-117)	2 (97-111)	2 (97 – 105)	4 (97 – 107)	4 (97 – 107)	2 (93 – 99)	-	-
p*Cl*A138	5 (174-186)	6 (178-192)	5 (166-188)	1 (176)	1 (170)	-	3 (180 – 202)	8 (180 – 200)	6 (178 – 194)	3 (178 – 182)	4 (174 – 182)	3 (176 – 182)
p*Cl*B11	6 (119-214)	4 (187-205)	-	-	7 (193-238)	-	3 (199 – 226)	-	-	-	-	1 (205)
p*Cl*D108	4 (218-232)	6 (210-234)	11 (214-254)	10 (234-278)	-	2 (242-250)	8 (198 – 234)	-	-	3 (222 – 246)	-	9 (222 – 266)
p*Cl*D122	7 (114-148)	7 (22-146)	10 (94-142)	-	-	3 (150-154)	-	3 (98 – 122)	3 (106 – 130)	-	-	-
p*Cl*D118	8 (187-215)	6 (191-211)	19 (187-259)	9 (249-293)	11 (191-239)	2 (169-173)	11 (201 – 257)	6 (183 – 211)	-	3 (173 – 181)	1 (177)	2 (171 – 175)
p*Cl*D7	7 (96-124)	7 (92-116)	8 (80-116)	4 (92-104)	4 (99-131)	3 (152-160)	2 (116 – 120)	4 (116 – 128)	4 (116 – 128)	4 (130 – 142)	4 (104 – 120)	-
p*Cl*D105	4 (83-111)	5 (83-115)	8 (83-115)	4 (115-127)	3 (129-137)	-	3 (117 – 125)	6 (109 – 131)	4 (115 – 127)	3 (111 – 119)	5 (115 – 135)	8 (83 – 119)
p*Cl*D109	5 (168-184)	5 (168-188)	4 (144-180)	1 (144)	4 (202-214)	3 (156-164)	4 (192 – 204)	3 (188 – 196)	3 (188 – 196)	2 (168 – 176)	-	1 (160)
p*Cl*D114	4 (124-136)	4 (120-132)	10 (116-156)	7 (140-162)	6 (124-144)	-	5 (124 – 140)	6 (120 – 144)	5 (120 – 148)	5 (108 – 128)	5 (120 – 144)	5 (120 – 140)
p*Cl*D112	4 (167-179)	8 (151-191)	-	8 (179-227)	2 (171-191)	-	-	3 (163 – 175)	-	6 (169 – 237)	-	-
p*Cl*5*	5 (164-190)	3 (164-172)	6 (154-170)	3 (160-172)	4 (152-170)	3 (152-160)	5 (160 – 176)	-	-	-	6 (158 – 214)	-
p*Cl*13*	6 (118-132)	7 (116-130)	6 (118-134)	2 (116-120)	2 (112-114)	2 (112-122)	3 (118 – 122)	4 (122 – 128)	4 (120 – 128)	6 (120 – 134)	2 (120 – 124)	5 (110 – 120)
p*Cl*8*	5 (128-144)	5 (128-144)	9 (132-164)	8 (128-164)	6 (120-144)	2 (136-140)	5 (112 – 140)	6 (112 – 212)	5 (112 – 132)	2 (108 – 116)	3 (120 – 140)	-
% of loci variable	100	100	85	65	65	60	80	75	65	75	50	50

For each locus (GenBank accession numbers: GQ358643- GQ358661), we list the number of alleles and size range, ( ). " – " indicates unsuccessful amplification of PCR product, *primers (GenBank accession numbers AY775326 – 29) redesigned for this study McInnes *et al.* 2005.

***Cover letter**

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1st of August, 2009

The Editor,

Conservation Genetics Resources

Dear Sir/Madam,

Please find attached a manuscript entitled "**Characterisation and cross-species utility of 20 microsatellite markers for population and forensic applications in the endangered Carnaby's Black-cockatoo, Calyptorhynchus latirostris**" which we would like to submit as a technical note for consideration in *Conservation Genetics Resources*.

As a short communication, we believe the manuscript will add to the increased interest that cockatoos and parrots are contributing to our understanding of conservation and as animals that have a high profile in the illegal trade in wildlife.

This manuscript has never been previously submitted, published, nor is it being considered for publication in any journal other than *Conservation Genetics Resources*.

Kind regards

P. Spencer

(on behalf of both authors)